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The purposes of the research are to elucidate the metabolism and toxicity of tricothecene mycotoxins, identify and synthesize their active metabolites and identify and synthesize compounds to block or reduce their toxicity.

An efficient procedure for purification of anguidine has been developed to provide uniformly pure samples for all phases of the program. Tritiated anguidine has been prepared for use in the metabolic studies, and metabolites have been synthesized for use as standards

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FIGURE 2

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in identification of metabolites. Synthesis of nivalenol is proceeding.

Conditions have been established for study in vitro of hepatic microsomal metabolism and for isolation, identification and quantitation of metabolites of anguidine. Metabolism occurs rapidly in the system; the three metabolites detectable by fluorescence on TLC are present within 15 minutes. Two have been provisionally identified as acetoxyscirpenediol and scirpenetriol. Preparation of larger amounts of the metabolites and HPLC separation and structure identification of them and of additional metabolites are in progress. In vivo studies of distribution and metabolism of ^{3}H-anguidine after parenteral exposure are being performed in mice.

Toxic effects of anguidine given in lethal or sublethal doses to mice and rats by gavage, parenteral or topical cutaneous application have been studied by histopathologic and hematologic examination. The LD_{50} has been measured, and sublethal doses that give consistent effects on the hematopoetic and lymphoid systems have been established. The information is being used to detect and quantify the effects of potentially protective compounds such as the radioprotectants and a series of thiazolium salts. Several of the salts have been synthesized and tested. They have not shown any protective activity even when given at near-toxic doses. Thiamine also was tested because of its structural similarities to the thiazolium salts, but it had no effect.

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TRICOTHECENES MYCOTOXIN STUDIES

Annual Summary Report

G.H. Buchi, M.A. Marletta, P.M. Newberne,
A.E. Rogers, W.R. Rousch, G.N. Wogan

September, 1983

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Massachusetts Institute of Technology
Cambridge, MA 02139

Summary

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Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Table of Contents

Segment 1A Metabolism

M.A. Marletta
J. Recchia

1. In Vitro Studies

The studies to date have been directed towards producing metabolism of anguidine by incubation with phenobarbital-induced rat liver microsomes and determining how many metabolites exist. Progress has been made in several areas. The optimum conditions for the microsomal incubations have been developed along with clean-up procedures to remove microsomal components from the extraction mixtures. Metabolites have been separated by TLC, and preliminary identifications have been made.

Optimum conditions for the incubation of anguidine with phenobarbital-induced microsomes have been developed. Incubations are carried out in 0.1 M PO_4, pH 7.5 at 37°C with shaking to keep the solution well aerated. A regenerative NADPH system consisting of NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase was utilized to maintain NADPH at saturating levels. Incubations were conducted from 5 min. to 2 hrs., and the metabolites extracted 3 times with 2 ml ethyl acetate, the organic layers combined, and then evaporated under reduced pressure. The metabolites were dissolved in 2 ml of acetonitrile. Microsomal lipid components were extracted from the ethylacetate twice with 2 ml of hexane. The acetonitrile layer was saved and dried down for further analysis.

Metabolites are isolable by 2-dimensional TLC on silica plates. The solvent systems used were 0.001% HoAc/MeOH and $CHCl_3$/acetone (3:2) for the first and second dimensions, respectively. Those metabolites containing the 12,13 epoxy group were detected as fluorescent spots by the procedure of Sano et al (J. Chromatography 235:257-65, 1982). The fluorophore obtained is thought to be a stable napthylidine derivative which results from the N-alkylation of nicotinamide with the epoxy group, and subsequent condensation of this product with 2-acetylpyridine in the presence of base.

The presence of metabolites can be detected after 5 min of incubation with the microsomes. A total of 3 fluorescent spots, other than anguidine itself, were observable after 2 hrs.. The detection limit of this technique is 100 ng/spot. These spots are not visible in control experiments in which incubations were conducted without anguidine.

A preliminary identification of two of the spots has been made by comparison of their R_f-values with that of standards obtained from Dr. Roush's laboratory. Acetoxyscirpenediol and

scirpenetriol were in very good agreement with the unknowns. The total number of metabolites will be determined by HPLC with radioactivity detection.

Suitable HPLC conditions for the separation of scirpenetriol and acetoxyscirpenediol from anguidine have been developed. Isolation, under isocratic conditions, is accomplished using a 40% MeOH in H_2O solvent with a u-Bondapak C_{18} column at a flow rate of 1.5 ml/min. A refractive index detector is used. As expected, the more polar metabolites elute first in the order scirpenetriol, acetoxyscirpenediol, and anguidine with retention times of 4.4, 11.6 and 26 minutes, respectively. Incubations with radioactive anguidine will permit the quantitation, as well as the detection of all metabolites.

We obtained ^{3}H-anguidine from Dr. Roush's laboratory with a specific activity of approximately 13.4 mCi/mmole. The radioactive material was found to be stable when stored cold in 100% EtOH. It was not stable as a solid. The recovery of metabolites from the incubation mixture using previous extraction techniques was found to be 45% of the total radioactivity. An alternative procedure produced 85-90% recovery of metabolites. Upon termination of the incubation, NaCl was added until saturation, and then the metabolites were extracted twice with 2 ml of ethyl acetate. The ethyl acetate is not evaporated to dryness because of the instability of ^{3}H-anguidine. The hexane extraction was not performed because of this.

The metabolites are best isolated by reverse phase HPLC with a MeOH gradient. Figure 1 is the chromatogram of a 45 min. incubation. A flow rate of 1.5 ml/min is used and 1.5 ml fractions are collected and counted by scintillation. Unmetabolized anguidine elutes at 55% MeOH (25 minutes). Under these conditions, all of the starting material is metabolized after a 45 min. incubation. Table 1 shows the approximate yield of each metabolite.

Table 1

A	3%
B	41%
C	2%
D	47%
E	7%

Preliminary work shows that the retention time of product B, under the isocratic conditions given above, agrees with that of scirpenetriol. Product C or D may be acetoxyscirpenediol. Products B and C were observed in incubations performed without NADPH, conditions under which cytochrome P_{450} is inactive. (No products were observed in control incubations of anguidine without microsomes). However, identifications are tentative

since the ratio of products changes with time during storage. Also, further work is necessary to confirm the purity of each product. Structural analysis of the metabolites will be done by GC/MS.[a]

Microsomes contain other enzymes, besides cytochrome P_{450}, that are involved in the detoxication of xenobiotics. Examples of these are epoxide hydrolase[b] and carboxylesterase.[c] These enzymes exist as isoenzymes. Phenobarbital will induce epoxide hydrolase, as well as a few carboxylesterases. These isoenzymes have different substrate specificities, therefore, we would expect the metabolic profile to change when using uninduced or β-naphthoflavone-induced microsomes. The observation of metabolites in the absence of P_{450} activity suggests that anguidine is a substrate for the isoenzyme(s) of epoxide hydrolase and/or carboxylesterase. This point will be investigated further.

Future work will be directed towards purification of large enough quantities of metabolites for GC/MS analysis, as well as the development of suitable derivatization procedures and isolation conditions for gas chromatography.

[a]Rosen, R.T. and Rosen, J.D., Biomed. Mass Spectrom, 9, 443, 1982.
[b]Oesch, F., in Enzymatic Basis of Detoxification, Vol II, Chapter 16, 1980.
[c]Heymann, E., in Enzymatic Basis of Detoxification, Vol II, Chapter 15, 1980.

Figure 1

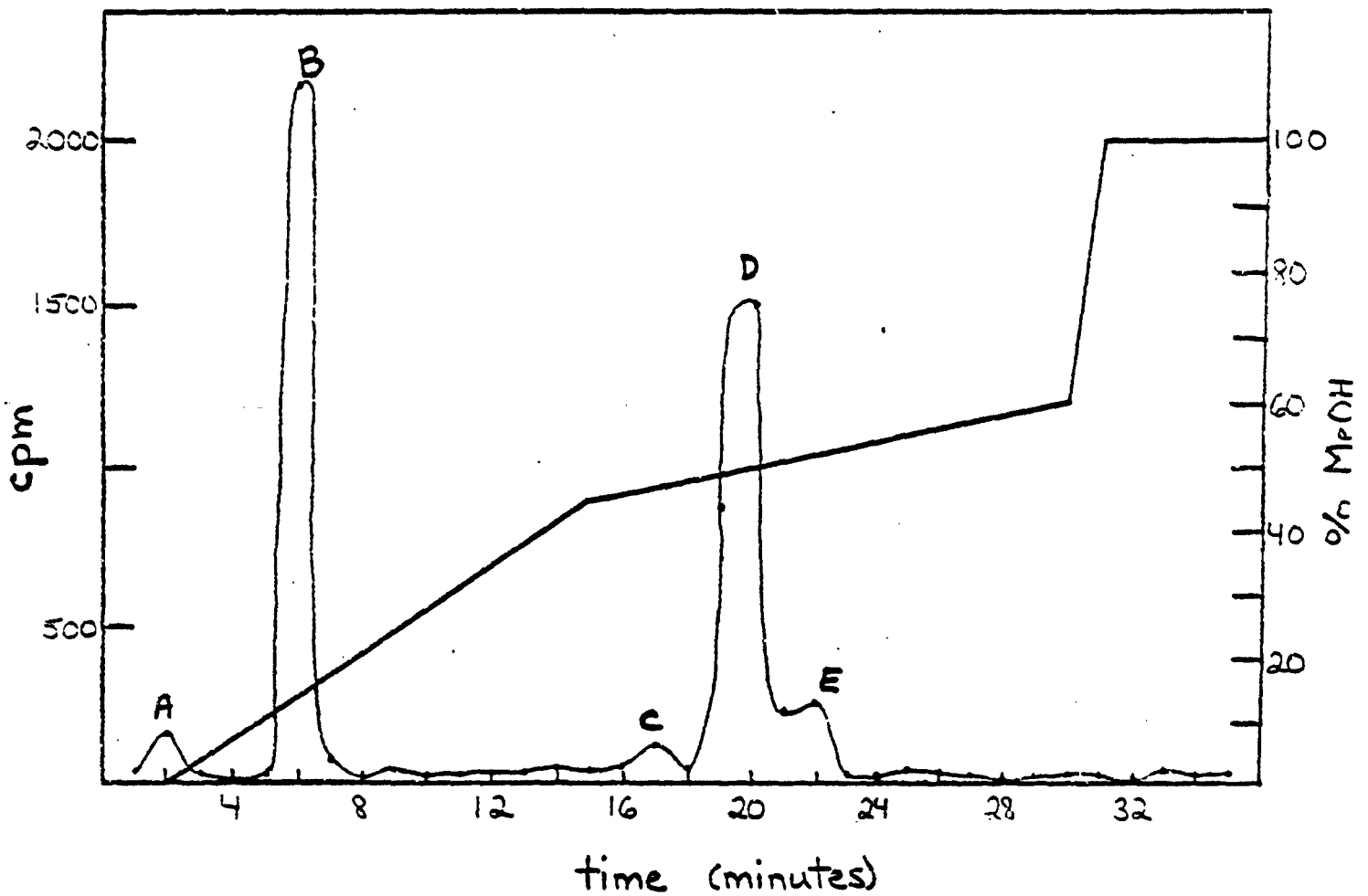


Fig. 1

Segment 1B Metabolism

Gerald N. Wogan
William F. Busby, Jr.

1. Preliminary studies are in progress to validate the counting procedures and to ensure maximal recovery of administered [^{3}H]-anguidine. For this purpose 5-week-old male CD-1 mice (25-30 gm) are being given 10 uCi of [^{3}H]-thymidine i.p. and we are taking the following organs and excreta for counting 2 hrs. later: lungs, heart, liver, stomach, small intestine (divided into 3 equal sections corresponding to the duodenum, ileum, and jejunum), cecum, abdominal fat, kidneys, spleen, bladder, testes, brain, femurs (bone marrow), skin, pooled residual organs (esophagus, thymus, trachea, pancreas, etc.), carcass, blood, urine feces, and contents of the gastro-intestinal tract.

2. In other studies we have designed non-occlusive skin barriers for percutaneous absorption studies in 5-week-old male CD-1 mice (25-30 g) and Fischer rats (90-110 g). The barriers remain intact on the animals for at least 7 days and permit application of at least 35 ul of solvent or suspension to a 1.2 x 1.2 cm application site on the dorsum of the rat and 10 ul to a 0.65 x 0.65 cm site on the mouse.

Using Na fluorescein as a fluorescent indicator in preliminary studies with 5-week-old rats, no loss of material from the application site was found as measured by fluorescence in urine, and cage and animal washings.

Rats treated with anguidine in DMSO were dead or moribund within 18 hours, after developing diarrhea and epistaxis. At necropsy pulmonary and gastrointestinal hemorrhagic necrosis were found. Rats treated with anguidine in acetone survived the 7 days of the experiment. At necropsy the skin at the application site was inflammed and the spleen was enlarged.

Segment 2A Chemistry

W.R. Roush
T.T. Caggiano

We proposed originally to participate in four phases of this program:

(1) Preparation of Radiolabelled Trichothecenes. Radiolabelled anguidine and nivalenol are required for metabolism studies to be carried out by Professors Wogan and Marletta. Because nivalenol is not available from commercial or private sources, preparation of radiolabelled nivalenol requires that a synthesis from anguidine (or some other available trichothecene) be developed.

(2) Structure Determination of In Vivo and In Vitro Generated Metabolites. This work will be performed in collaboration with Professors Wogan and Marletta, who will generate and isolate metabolites during their studies.

(3) Synthesis of Metabolites. Metabolites will be synthesized in order to confirm structure and also to prepare sufficient quantities for biological evaluation.

(4) Synthesis of Trichothecene Analogues. We proposed to synthesize various nuclear analogues of anguidine in order to probe the mechanism of action. This area is of lower priority than (1)-(3), above, and as a consequence, studies on this problem have not yet been initiated.

Results

1. Purification of Anguidine

A potential problem for this program as a whole is the availability of anguidine. An initial 10 g supply was obtained from the National Cancer Institute, but this source is probably limited. Anguidine is commercially available from Myco-Lab Co., but is very expensive ($1275/g for 99% pure material). As an alternative, we purchased 2 g of "crude" anguidine from Myco-Lab ($450/g) in order to develop an efficient purification scheme. The procedure is outlined below.

A bottle received from Myco-Lab containing "1g-activity" of mycotoxin in approximately 25 mL of syrupy liquid (predominantly lipid-like materials) was transferred with the aid of 80 mL of $CHCl_3$ to a 500 mL round-bottom flask. This solution was diluted with 240 mL of CH_3CN and then extracted overnight with hexane (400 mL) using a continuous extractor. The CH_3CN layer was separated and concentrated in vacuo to give 2.98 g of brown foam containing anguidine. This material was chromatographed on a 6 inch x 60 cm column of silica gel using 3:1 EtOAc-hexane (1.3 L) followed by 700 mL of EtOAc as eluent (15-mL fractions collected). Fractions 17-28 containing anguidine by TLC analysis. These were combined, concentrated to give 1.2 g of crude anguidine, and crystallized from hexane-ether (two crops to give

835 mg of pure toxin, mp 162-163°C. An additional 540 mg (total yield, 1.37 g) of anguidine was obtained by chromatography and crystallization of the mother liquors.

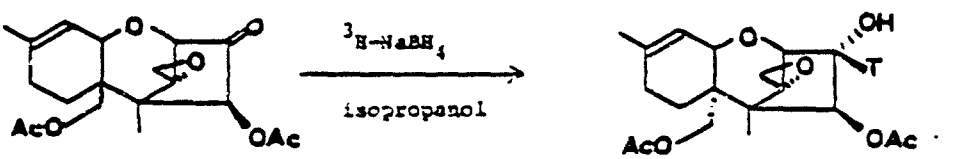
This procedure can be performed very easily within a one week period and is relatively efficient. Thus, we have purified a total of 2.2 g of anguidine from two "1 g" samples of crude mycotoxin. If additional quantities of anguidine are required in the future, we will purchase more of the crude mycotoxin and purify it according to this procedure.

We have found that anguidine is rapidly decomposed to the inactive apotrichothecene ring system by aqueous mineral acid. We have incorporated this into our safety protocol.

2. Synthesis of ^{3}H-Anguidine

We have worked out a procedure for tritium labelling of anguidine, and have prepared 30 mg of material with a specific activity of 180 m Ci/mmol. The procedure is briefly summarized below.[1]

Ketone 1 was prepared according to Doyle's published procedure by oxidation of anguidine with the reagent prepared from



1 ^{3}H-anguidine

trifluoroacetic anhydride and DMSO.[2] A troublesome step is the $NaBH_4$ reduction of 1 which gives a mixture of stereoisomers (approximately 9:1) together with some deacylation products. Purification of anguidine from this mixture by chromatographic methods is rather inefficient. Consequently, we prepared the ^{3}H-toxin by using isotopic dilution techniques. Thus, 19 mg of 1 was reduced with 0.43 mg (100 mCi, 8.8 Ci/mmol) of ^{3}H-$NaBH_4$ in 3 mL of dry isopropanol (room temperature, 24h). The crude product obtained after aqueous workup was diluted with 62 mg of cold anguidine. This sample was crystallized twice from ether-hexane to give 30 mg of pure ^{3}H-anguidine (180 mCi/mmol). The mother liquors were diluted with additional cold anguidine and crystallized as before to yield 20 mg of ^{3}H-anguidine (57 mCi/mmol).

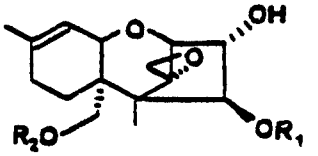
These crystalline samples were stored at 5°C under N_2. Over the course of one month, however, extensive decomposition of the

labelled DAS was noted by Dr. J. Recchia in Prof. Marletta's laboratory (HPLC/LSC analysis). TLC analysis showed two components more polar than DAS. This necessitated that the labelled DAS be repurified.

The samples were pooled and applied to two 20x20 cm x 0.25 mm silica gel preparative plates, which were then developed once with 5% MeOH in CH_2Cl_2. The anguidine-containing band was removed and washed with $CHCl_3$ (5x50 mL). The filtrate was concentrated in vacuo to give 40 mg of a white powder which was pure DAS by TLC analysis. This sample was dissolved in 9 mL of absolute EtOH and stored under N_2 in a -20°C freezer. HPLC/LSC analysis showed this material to be radiochemically pure. The specific activity was 13.4 mCi/mmol. This material is being used by Dr. Recchia and no decomposition has yet been noted.

3. Identification/Synthesis of Potential Metabolites

Professor Marletta has generated a number of anguidine metabolies using his in vitro metabolism system. Sufficient quantities for characterization by spectroscopic techniques have not yet been obtained, however. In order to assist his studies, we have prepared reference samples of a number of compounds which we expected would turn up in the spectrum of natural metabolites. In particular, samples of C.15-monoacetate (2b) and triol (2d) derivatives of anguidine were synthesized[3] by modifications of literature procedures.[6] Dr. Marletta has now identified 2b and 2d as metabolites of anguidine by chromatrographic comparison with the in vitro generated mixture. Syntheses of other poten-



2a $R_1 = R_2 =$ Ac (anguidine)
2b $R_1 =$ H, $R_2 =$ Ac
2c $R_1 =$ Ac, $R_2 =$ H
2d $R_1 = R_2 =$ H

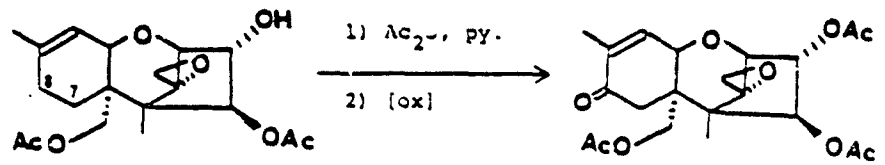
tial metabolites, including monoacetate 2c, will be performed in the coming grant year, as outlined in the Proposed Research section of this report.

Samples of 2b and 2d (ca. 25 mg each) have been submitted to Professor Newberne for eventual toxicity studies.

4. Progress Towards the Synthesis of Nivalenol

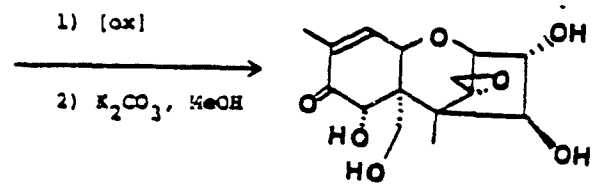
Our plan for the synthesis of nivalenol from anguidine is summarized in Scheme I. Procedures for effecting this conversion have been published, but the yields for two oxidation steps (at C.8 and C.7, respectively) are very low.[4,5] Thus, the development of an efficient synthesis of nivalenol from anguidine requires that alternative oxidation procedures be developed.

Towards this end we prepared triacetate 5 by acylation of anguidine (Ac_2O, pyridine)[6] and studied its oxidation under a variety of conditions (Table I). Noteworthy are the discovery of selective conditions for production of allylic alcohol 6, enone 4, and aldehyde 7 in fair to good yield. In addition, oxidation of 6 with pyridinium chlorochromate in CH_2Cl_2 affords enone 4 in high yield.[11] Thus, two different routes to enone 4 are now available. Studies on the conversion of 4 to nivalenol (see Scheme I) are in progress.



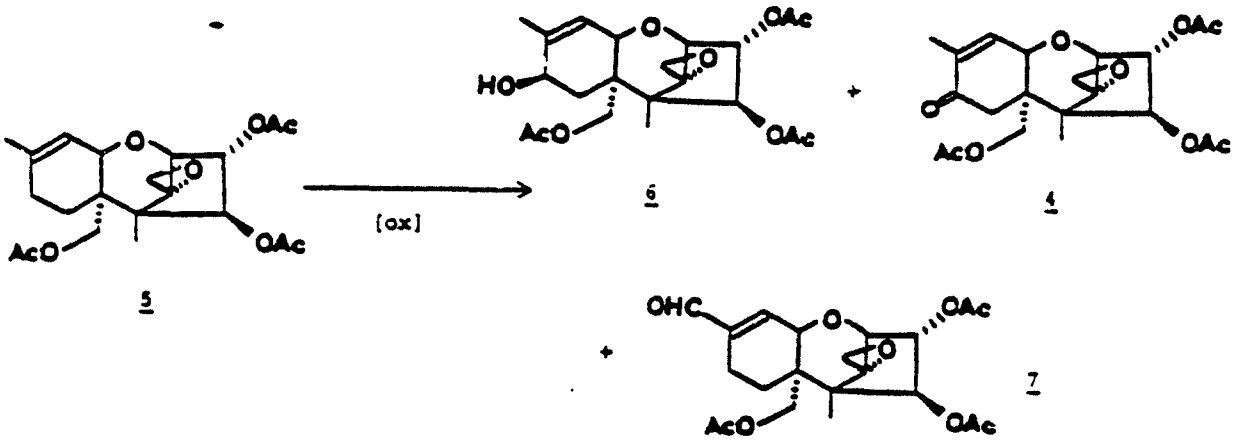
Anguidine

4



Nivalenol (3)

Table I - Allylic Oxidations of Triacetate 5



	Oxidation Conditions	Isolated Yield 6	4	7
1.[7]	SeO_2, HOAc, Ac_2O, 90°C	75%	-	-
2.[2a]	SeO_2, H_2O, dioxane, reflux	55%[a]	-	-
3.[8]	$PdCl_2$, AgOAc, HOAc, TeO_2, ^tBuOOH	-	70%	-
4.[9]	CrO_3, 3,5-dimethylpyrazole, CH_2Cl_2	-	41%[b]	-
5.[10]	CrO_3, pyridine, CH_2Cl_2	-	-	47%

---

[a] [illegible]% of 5 was recovered.

[b] 39% of 5 was recovered.

References

1. Some of the initial studies on the preparation of ^{3}H-anguidine were performed by Mr. A.P. Spada and Dr. W.K. Chong before Dr. Caggiano joined this program.

2. (a) Kaneko, T., Schmitz, H., Essery, J.M., Rose, W., Howell, H.G., O'Herron, F.A., Nachfolger, S., Huftalen, J., Bradner, W.T., Partyka, R.A., Doyle, T.W., Davies, J., and Cundliffe, E., J. Med. Chem. 1982, 25, 579. (b) We thank Dr. T. Doyle for providing the procedure for radiolabelling of anguidine prior to publication.

3. The preparation of 3b and 3d was performed by Mr. A.P. Spada.

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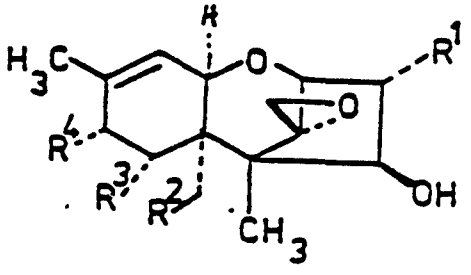
Segment 2B Chemistry

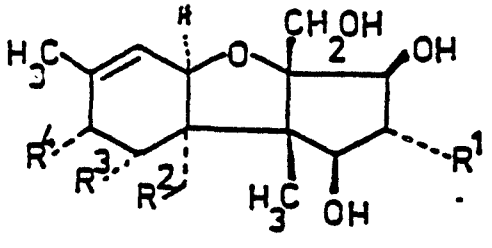
G.H. Buchi
G. Breipohl

Introduction. 12,13-Epoxytrichothecenes with the general structure 1 are terpenoid metabolites produced by various fungi.[1-4] These mycotoxins were involved in the outbreak of several human and animal mycotoxicoses[1,3,4] and have recently gained public interest, as they are believed to be used as chemical warfare agents in South-East Asia and Afghanistan.[5] The toxic effect is probably due to inhibition of protein synthesis in eukariotic cells and both the 12,13-epoxide and the 9,10-double bond are considered responsible for the toxicity of trichothecenes.[3] Reductive opening of the epoxide and reduction of the double bond result in a substantial loss of biological activity.[1,3]

Rearrangement of 12,13-epoxytrichothecenes to apotrichothecenes 2 and compounds with structure 3 is of interest as these products are not toxic.[3] For detoxification of 1 this transformation could therefore be of interest.



1



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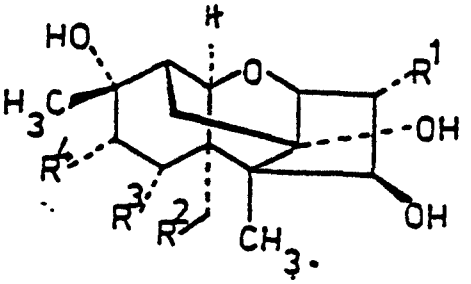

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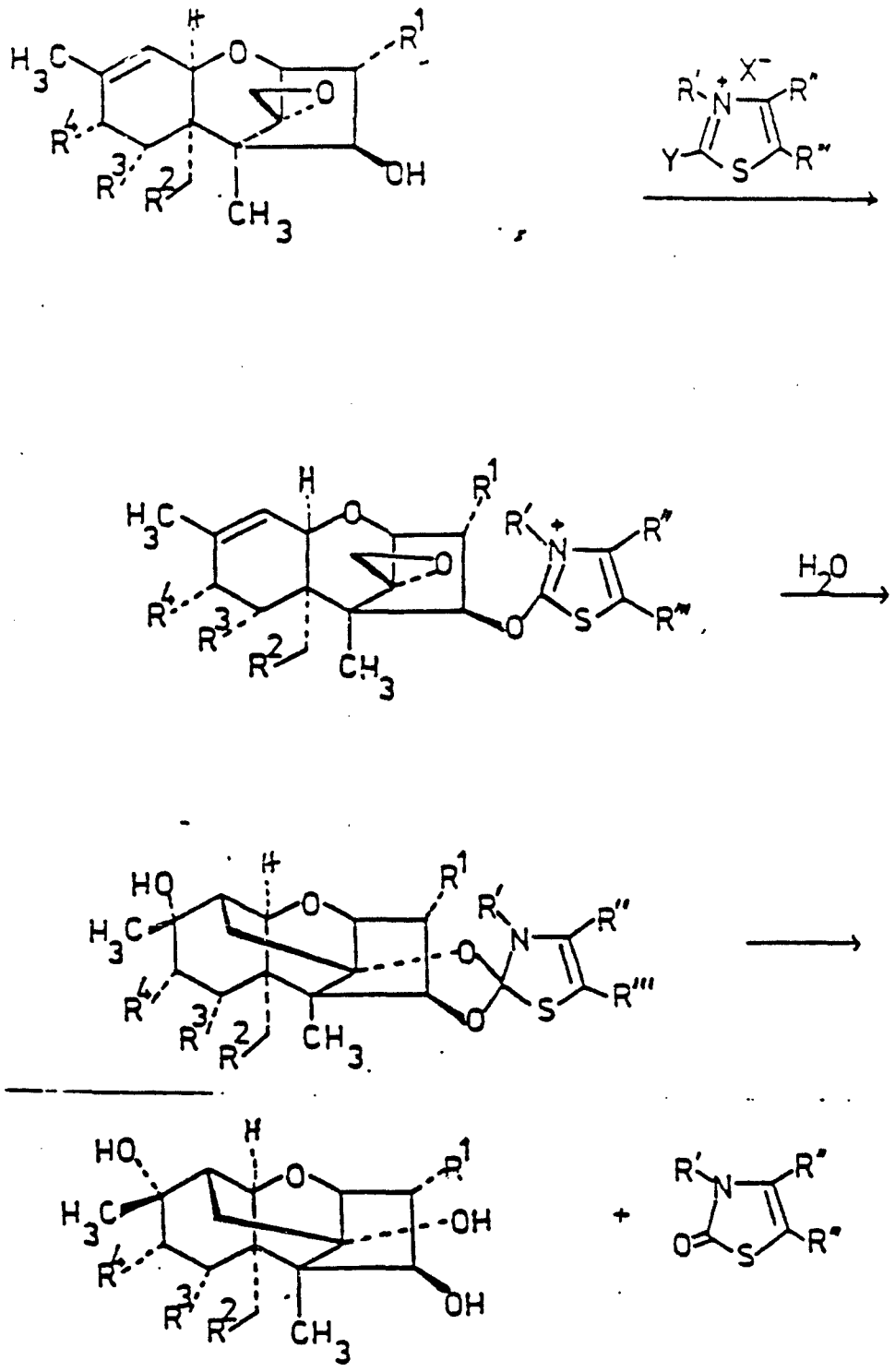
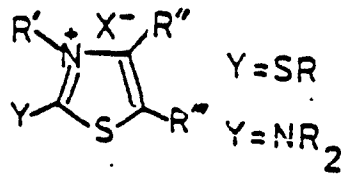


Scheme 1

Because the 12,13-epoxide group is rather resistant to intermolecular attack an intramolecular reaction which leads to rearrangement with participation of the double bond was sought after. We chose 2-substituted thiazolium salts 4 as electrophilic reagents in the hope that the readily accessible C_4-hydroxy group would combine with such salts. Expulsion of the leaving group Y would create a new electrophilic center which might induce a intramolecular rearrangement to 3 (Scheme 1). Indeed, some thiazolium salts are known to combine with nucleophiles, especially hydroxide ion.[6] Derivatives of thiamine (vitamin B_1) 5 became of special interest because the basic aminopyridine substituent could facilitate the reaction of the potential detoxicant with the secondary alcohol group at C-4 of a trichothecene.



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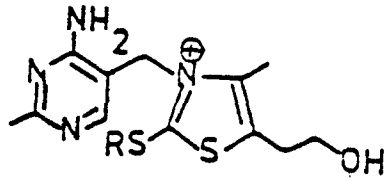
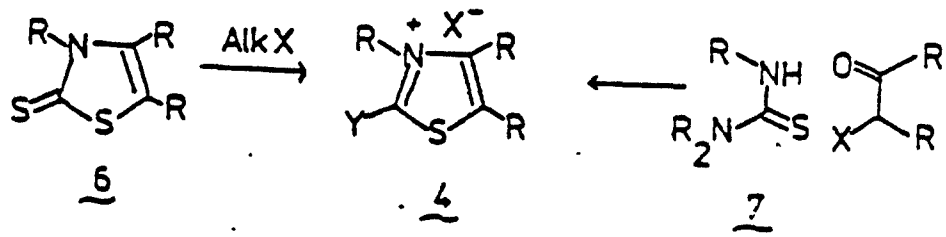


5

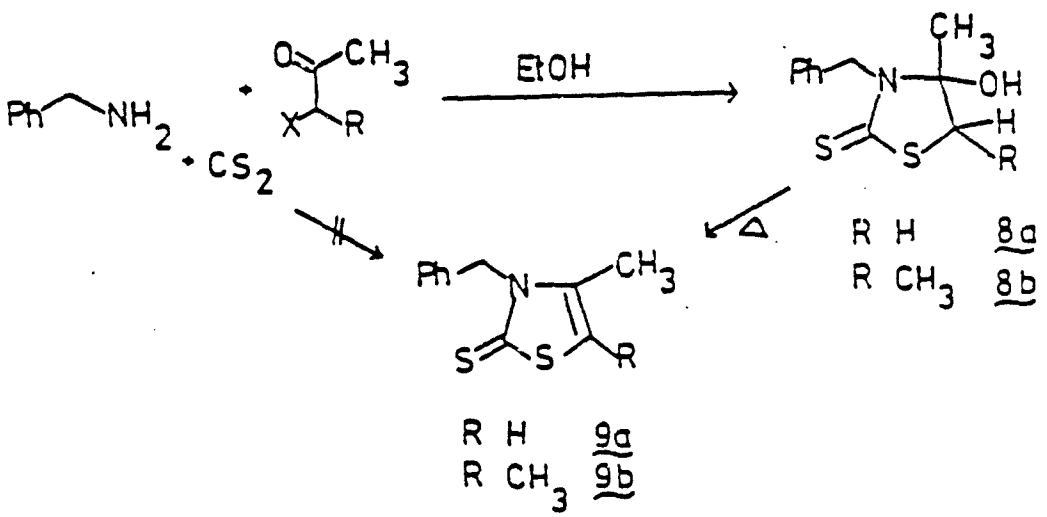
The thiazolium salts 4 should be accessible either by S-alkylation of the corresponding thiones 6 or by condensation of the corresponding thioureas 7 with -halogenoketones[6,7] (Scheme 2).

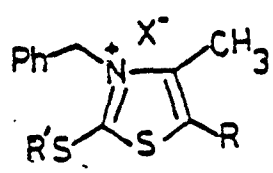


6 4 7

Scheme 2

For initial investigations 2-alkylthio-thiazolium salts 10 were synthesized.[6] When preparing the corresponding thiazoline thiones 9 according to the literature[6,8,9] the 4-hydroxy-thiazolidine thiones 8 were obtained instead. Intermediates of this type were described earlier by Lamon.[10] These compounds, however, lost water when heated above their melting point and formed the desired thiones 9 in good yield.



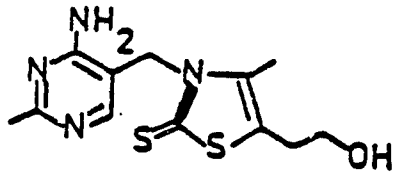


R	R'	X	
H	Me	I	10a
Me	Me	I	10b
H	Bnz	Br	10c
Me	Bnz	Br	10d

Reaction with excess alkyl halide without solvent at room temperature gave the thiazolium salts as crystalline products.[6] When thiamine thione 11 was treated with methyl iodide, however, the mixture became yellow and started to smell of methylthiol. TLC disclosed a complex reaction mixture which contained a compound with intensive blue fluorescence.

Possibly, the intermediate thiazolium salt 5 can react intramolecularly with explusion of methylthiol to form thiochrome

12 12,13 which indeed could be isolated from the complex mixture in small amounts. A similar reaction is probably involved in an industrial synthesis of thiochrome from 13 and 14 in the absence of solvent.[14]



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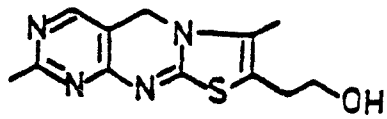

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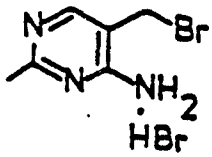

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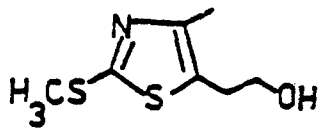

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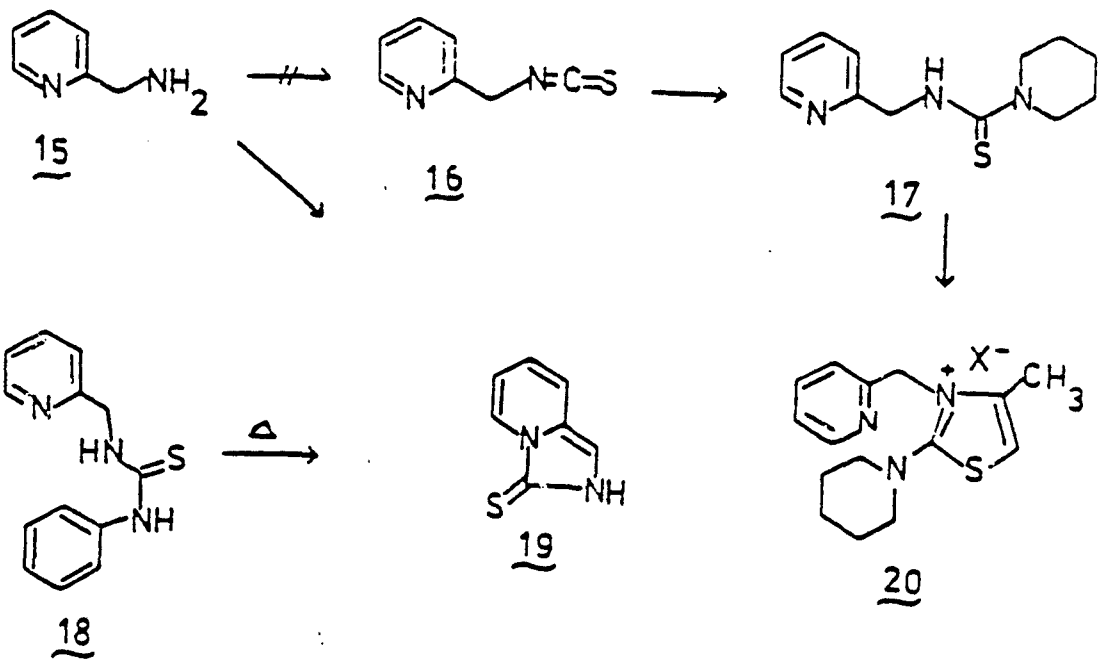
Because the proposed synthesis of 5 led to a complex mixture and because toxicological studies showed compounds 10 to be rather toxic (LD_{50} of 10a 50 mg/kg)[15] and enhancing the toxicity of trichothecenes, the synthesis of this compound was abandoned. The toxicity of 10 could be due to the alkylthio group and it seemed reasonable to change this substituent to a dialkylamino group. A basic nitrogen on the C-3 position of the thiazolium salt could facilitate the intermolecular reaction with a trichothecene as outlined above. It was decided to introduce a (2-pyridyl)methyl substituent, and the necessary thiourea 17 could be prepared from 2-isothiocyanotomethyl pyridine 16 by reaction with piperidine.

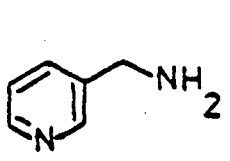
When attempting to prepare the isothiocyanate 16 by the method of Jochims,[16,17] we did not obtain 16 but a dark red solid which contained mainly dicyclohexylthiourea and another product. After separation its structure was determined to be imidazo(1,5-a) pyridine-3(sH)-thione 19 identical with the compound obtained by thermal decomposition of 18 as reported earlier.[18,19] The structure of 19 was confirmed by independent synthesis according to the literature.[18]

structure 19



15 16 17 18 19 20

When 3-aminomethyl-pyridine 21 was treated in a similar way[16,17] isothiocyanate 22 and thiourea 23 were obtained smoothly, but the latter combined with chloroacetone to give a complex mixture.



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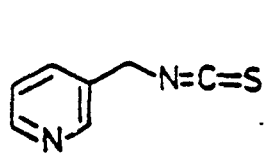

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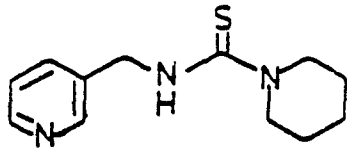
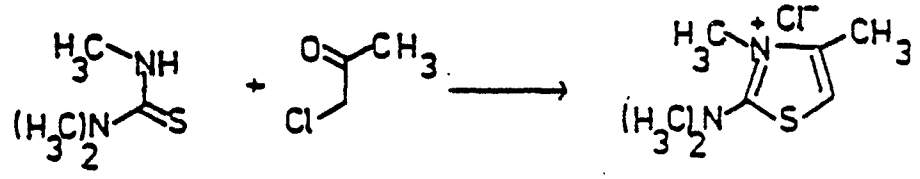


23

After these failures we decided to synthesize 2-dimethylamino-3,4-dimethylthiazolium chloride 25 which was originally prepared by Garreau[7] who did not report a detailed experimental procedure nor any spectroscopic data.

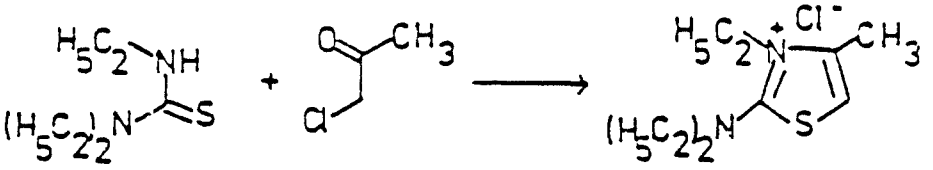
When an equimolar mixture of N,N,N'-trimethylthiourea 24 (prepared from CS_2, Me_2NH, $MeNH_2$)[20] and chloroacetone was heated without solvent to 80° it reacted vigorously to form a dark brown mixture, but by improving the workup procedure a hygroscopic product was obtained in crystalline form.



24 25

A similar reaction with N,N,N'-triethylthiourea 26 required higher temperature and longer reaction time to form 27 in moderate yield. Reaction of 28 and 30 with chloroacetone under similar conditions led to the adducts 29 and 31. Several attempts to eliminate H_2O led to decomposition of 29. Compound 32, however, could be obtained by dehydration of 31 with N,N'-carbonyldiimidazole.[21]

structure 31



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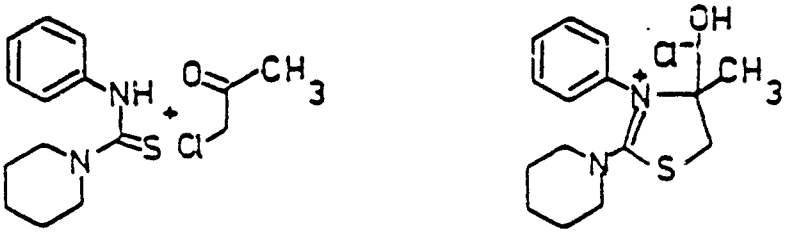

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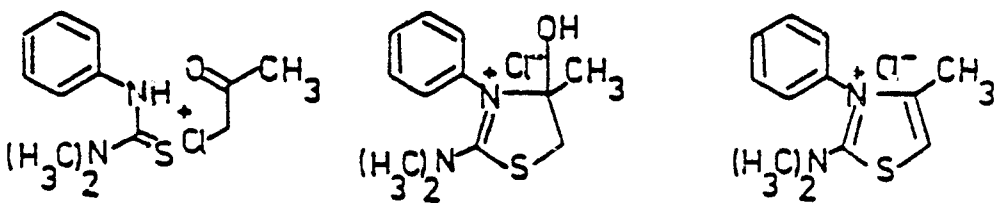
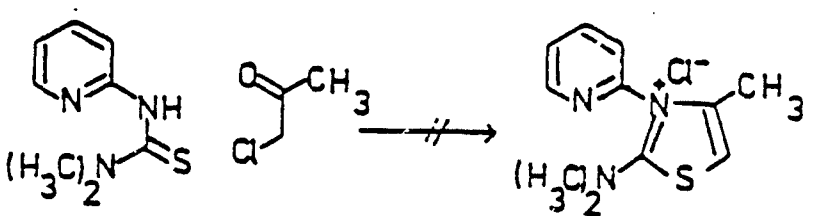
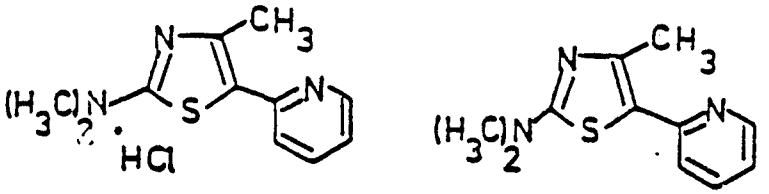


30 31 32



33 34



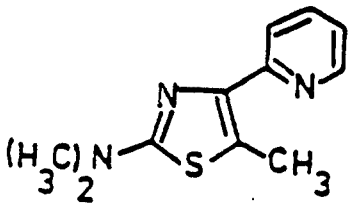
35 36

Finally, we attempted to prepare 34 by condensation of thiourea 33[22] with chloroacetone. When an equimolar mixture of the two compounds was heated to 50° a strongly exothermic reaction ensued giving a brown-yellow solid that crystallized from chloroform to give a yellow compound mp 226-228° (sublimes at 185°). Spectroscopic data of this new substance did not agree with those anticipated for compound 34. Infrared absorptions at 2550 and 1625 cm^{-1} indicated the presence of a tertiary amine salt and the mass spectrum with a parent peak at m/z 219 and intense peaks at m/z 38 and 36 (HCl) suggested the presence of a hydrochloride. Ultraviolet absorption at 310 nm was shifted to 37 nm in base and when a solution of the salt in water was treated with base a yellow oil separated which crystallized. Treatment of an etheral solution of the free base with gaseous hydrogen chloride gave the hydrochloride obtained in the original synthesis.

The reaction product is obviously a hydrochloride and as the free base was much more soluble in organic solvents NMR spectra were recorded for this compound. IH-NMR showed a methyl-singlet at 2.48 ppm, a singlet at 3.00 ppm (6 H, dimethylamino group) and multiplets at 6.7-7.0 (1 H), 7.15-7.6 (2 H) and 8.25-8.5 (1 H) ppm which are due to a 2-pyridyl group. ^{13}C-NMR showed signals at 17.74 (quartet), 39.45 (quartet), 119.18 (doublet), 119.27 (doublet), 120.96 (singlet), 135.66 (doublet), 147.16 (singlet), 148.78 (doublet), 152.31 (singlet) and 168.76 (singlet) ppm. In the off-resonance spectrum the signal at 152.31 ppm was broadened by long range coupling and it was therefore assigned to C-2 of the pyridyl substituent. The four doublets were assigned to the remaining carbon atoms on the pyridine ring. The quartet at 17.74 ppm was assigned to an aryl-methyl group and the quartet at 39.45 ppm to a dimethylamino group. The remaining three signals are due to a trisubstituted thiazole. These findings are best accomodated by structure 36.

Regioisomer 37 was excluded by the following considerations. The large bathochromic shift observed in the transformation of the salt 35 to the free base 36 is better accomodated by structure 36 rather than 37 because the nitrogen atom of the dimethylamino is conjugated with the pyridine ring only in the former structure. A similar UV behavior was observed with 2-amino-4-methyl-5-phenyl-thiazole 38 whereas its regioisomer 39 absorbs at shorter wavelengths than its hydrochloride.[23] Furthermore the signal due to the methyl group in the ^{13}C-NMR spectrum should appear at higher field if it were located at C-5 of the thiazole ring.

A possible mechanism for the formation of 35 is presented in scheme 3. Thiourea 33 combines with chloroacetone by substitution on sulfur. Adduct 40 could now cyclize, possible with assistance from the released hydrogen chloride to produce the spiro intermediate 41. Ring opening with generation of a new pyridine 42 followed by cyclization and dehydration would lead to the thiazole 35.



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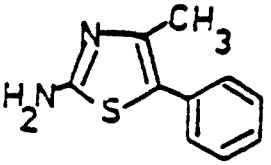

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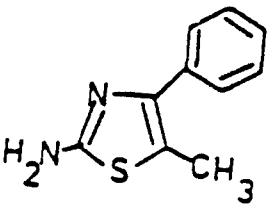

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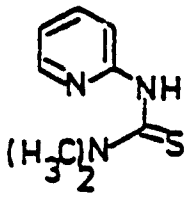

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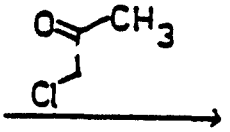

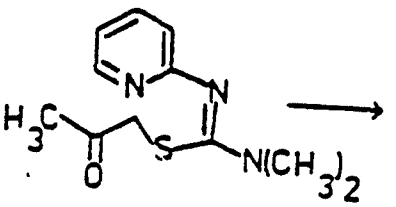

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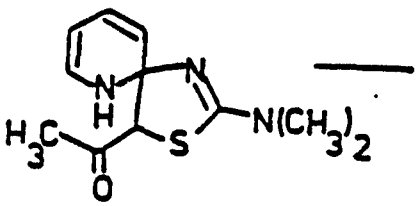

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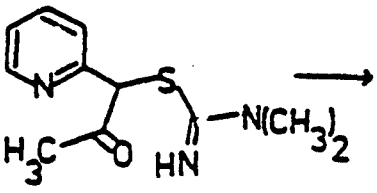

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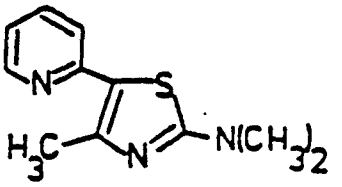

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Segment 3 Toxicology

P.M. Newberne, A.E. Rogers,
K.M. Nauss, J. deCamargo,
P. Punyarit, and S. Riengrojpitak

We have:

1) Measured the LD_{50} of anguidine given parenterally, by gavage or by inhalation in mice.

2) Examined the dose- and time-related effects on lymphoid organs, bone marrow and peripheral blood counts of anguidine administered intraperitoneally to mice and chosen a dose of anguidine that gives a measurable, consistent response that can be used as a test regimen for examining effects of the potentially protective thiazolium and radioprotective compounds.

3) Assayed the toxicity of several potentially protective compounds in mice and made initial studies of effects of certain of the potentially protective compounds on anguidine lethality and tissue damage in mice.

4) Prepared a detailed protocol for safe handling of the tricothecene mycotoxins in the laboratory.

Materials and Methods

Male CD-1 mice, 21-34 g, were given anguidine dissolved in aqueous DMSO. They were bled from the retroorbital plexus at the stated times after treatment and necropsied. Complete blood counts were performed using standard methods; complete necropsies were performed; tissues were fixed and processed using standard methods; detailed histological evaluation is in progress and is partially completed.

1. Studies in Mice Given Lethal Doses of Anguidine

The LD_{50} in male CD-1 mice, 96 hours after anguidine is administered intraperitoneally in 10% DMSO in water, is 20 mg/kg with 95% confidence limits 17.4-24.6. The LD_{50} of intraperitoneal DMSO in mice in our laboratory is approximately 14 g/kg; we have seen no evidence of toxicity at 1 g/kg, the average amount given. In mice examined 2-4 days after intraperitoneal doses of 15-29 mg/kg of anguidine, we have found mucosal or transmural necrosis of small and large intestine with little or no evidence of regeneration, destruction of both red and white pulp of the spleen, of germinal centers in lymph nodes and thymic cortex and partial or complete arrest of spermatogenesis. In mice given the highest doses, respiratory tract necrosis also was found. Tissues from animals that survived and were examined eight days after treatment were normal in most cases. Hematologic studies showed an early leukocytosis and hemoconcentration followed by leukopenia and anemia in surviving animals. The white blood cell count was markedly elevated at 6 hours from an average of 7000 cells/ul to an average of 42,000 cells/ul. All cell types were affected. Twenty-four hours after treatment the white cell counts were

within normal limits or slightly decreased with an increase in the neutrophil/lymphocyte ratio. At 72 hours the counts had decreased to an average of 2000 cells/ul, and the neutrophil/lymphocyte ratio was normal. The hematology profile was normal by day seven.

The LD_{50} at 72 hours after administration of anguidine into the upper gi tract is 15.5 mg/kg with 95% confidence limits of 13.5-17.8. It is, therefore, slightly lower than the LD_{50} for intraperitoneal administration. Gross and histologic changes are the same as were described following ip exposure.

2. Dose and Time-Related Effects of Anguidine Administered Intraperitoneally

Anguidine, 15 mg/kg, a dose that is lethal to approximately 5% of mice, caused a significant elevation of the wbc within 1 hour which persisted through 8 hours and then dropped to a nadir at 2-3 days; counts returned to normal by 5-7 days (Table 1). The differential count showed a shift from the normal ratio, approximately 75% lymphocytes and 25% polymorphonuclear leukocytes, to the reverse at 24 hours and then returned to normal. The hematocrit was significantly reduced at 24 hours and returned to normal by 7 days.

Anguidine, 10 mg/kg, a dose that is not lethal to mice, had similar but less marked hematologic effects with a drop in wbc at 24 or 48 hours and a return to normal range by 5 days. This dose did not cause a significant drop in hematocrit. DMSO had no effect on the blood counts (Table 1).

Histologic evaluation of lymphoid and hematopoetic tissues showed a clear dose response at 24 hours (Table 2). A dose of 1 mg/kg had essentially no effect. A dose of 5 mg/kg caused focal necrosis and cell depletion in thymus, spleen and bone marrow, and higher but sublethal doses had marked effects. Mice given 15 mg/kg revealed significant damage beginning within 1 hour, and largely repaired by 1 week (Table 3). In mice given 10 mg/kg there was damage at 1 and 2 days with progressive repair over the following week (Table 4). Cell populations were not entirely reconstituted in spleen and thymus even at 7 days.

3. Toxicity of Inhaled Anguidine

Mice were exposed to nebulized anguidine in DMSO for 15-35 minutes. The LC_{50} is approximately 11.9 mg-min/l, which corresponds to a dose of less than 11.3 mg/kg (Table 5, Fig. 1).

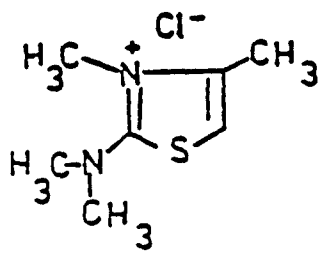
3. Toxicity of Potentially Protective Compounds

The monomethyl iodide thiazolium salt (see compound 10a in Prof. Buchi's segment) had an ip LD_{50} of approximately 70 mg/kg,

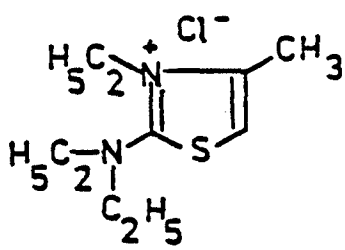
with 95% confidence limits of 48-95 mg/kg. An ip dose of 40 mg/kg, which resulted in no deaths when administered alone, was given 15 minutes before administration of 24 mg/kg anguidine, a dose which, given alone in that experiment, killed 50% of recipients. All mice given both compounds died, leading to the conclusion that the thiazolium compound potentiated rather than blocked anguidine toxicity.

Thiamin was tested in the same system. The approximate ip LD_{50} for thiamin was 200 mg/kg. We gave a dose of 100 mg/kg, which, given alone, was not lethal to mice, immediately after or 15 or 30 minutes before administration of an LD_{50} of anguidine. Thiamin had no effect on anguidine toxicity.

Two additional thiazolium compounds were studied. In mice given Compound V (below) there were no deaths at 25 mg/kg, 75% mortality at 50 mg/kg and 100% mortality at higher doses. For Compound VI (below) the respective figures are 50 and 100 mg/kg. WR2721 also was tested; there were no deaths in mice given 500 mg/kg or less; 3 of 4 mice given 1,000 mg/kg died.



(V)



(VI)

Groups of mice were given 10 mg/kg anguidine ip and one of the thiazolium salts, WR2721 or thiamin. The dose of each compound was approximately 25% of the (approximate) LD_{50}; the compounds were given ip as a single dose either 30 minutes before or 30 minutes after anguidine or both 30 minutes before and 30 miutes after anguidine. Mice were bled for hematologic studies and necropsied 48 hours after anguidine administration. Anguidine induced the characteristic decrease in wbc in all groups, and no evidence of protection by any of the compounds could be discerned. Histologic evaluation of the tissues is in progress.

Table 1. Effects of Anguidine on Peripheral White Blood Cell Counts in Male CD-1 Mice

Time After Administration of Anguidine	Anguidine Dose (mg/kg body wt., average ± SEM)				
	0	1	5	10	15
0					6520 ± 960
1 hours					26,500 ± 780
2					38,960 ± 1040
4					25,630 ± 840
6					24,310 ± 7650
8					18,190 ± 7410
1 day	9300 ±740	9010 ±920	4690 ±730	5250 ± 560	5160 ± 1130[a,b]
1 day	8558 ±647			8695 ± 1719	
2				5195 ± 489	
3				4819 ± 778	
4				6625 ± 469	
5				7765 ± 786	
6				10,667 ± 1235	
7				10,074 ± 984	

[a]Differential showed marked shift from 74% lymphocytes and 21% polymorphonuclear leukocytes in controls to 38% and 61%, respectively, in mice given 10 mg/kg and 35 and 64% respectively, in mice given 15 mg/kg.
[b]Hematocrit was reduced in these rats.

Table 2. Dose Response of Lympho-hematopoetic Organs to Anguidine: Lesions at 24 Hours

Anguidine Dose	1 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
Thymus	Cell Depletion (1/2)[a]	Necrosis (1/5) + Cell Depletion	Necrosis (12/12)	Necrosis (12/12)
Lymph Node	Normal	Normal	Necrosis (3/12)	Necrosis (1/12)
Spleen	Cell Depletion (1/2)	Cell Depletion (3/5)	Necrosis (4/12) + Cell Depletion	Necrosis (10/12)
Bone Marrow	Cell Depletion (1/2)	Cell Depletion (3/5)	Necrosis (8/12)	Necrosis (11/12)

[a] Number of mice affected/number examined.

Table 3. Time Response of Lympho-hematopoetic Organs to Anguidine, 15 mg/kg

	Hours		Days			
	1	6	1	3	5	7
Thymus	Cell Depletion (4/4)[a]	Necrosis (5/5)	Necrosis (12/12)	Necrosis (4/5)	Cell Depletion (3/3)	Normal
Lymph Node	Necrosis (4/4)	Necrosis (4/5)	Necrosis (1/12)	Normal	Normal	Normal
Spleen	Necrosis (1/4)+ Cell Depletion	Necrosis (5/5)	Necrosis (10/12)	Cell Depletion (1/5)	Normal	Cell Depletion (3/10)
Bone Marrow	Necrosis (4/4)	Necrosis (3/5)	Necrosis (11/12)	Necrosis (1/5)	Normal	Normal

[a]Number of mice affected/number examined.

Table 4. Time Response of Lympho-hematopoetic Organs to Anguidine, 10 mg/kg

Days	1	2	3	4	5	6	7
Thymus	Necrosis (12/12)[a]	Necrosis (3/4)	Cell Depletion (3/4)	Cell Depletion (2/4)	Cell Depletion (2/3)	Normal	Normal
Lymph Node	Necrosis (3/12)	Normal	Normal	Cell Depletion (2/4)	Cell Depletion (1/3)	Normal	Necrosis (2/7)
Spleen	Necrosis (4/12) + Cell Depletion (11/12)	Cell Depletion (4/4)	Normal	Cell Depletion (3/4)	Cell Depletion (2/3)	Cell Depletion (3/3)	Necrosis (1/7) + Cell depletion (4/7)
Bone Marrow	Necrosis (8/12)	Fibrosis (3/4)	Fibrosis (2/4)	Fibrosis (3/4)	Cell Depletion (1/3)	Normal	Normal

[a]Number of mice affected/number examined.

Table 5. Exposure of Mice to Anguidine by Inhalation

A. EXPOSURE CONDITIONS

(Concentration of Anguidine in DMSO = 67 mg)

A Weight Lost From Nebulizer During Run	B Anguidine Generated During Run A x 67 mg	C Duration Of Run	D Total Air-flow During Run 20.5 xc	E Nominal Concentration of Anguidine in Air B/D mg/L	F Concentration X Time CXT mg-min./L
1.27 (g)	85.09	15	307.5	.276	4.14
2.89	193.63	20	410	.472	9.44
3.15	211.05	25	512.5	.412	10.3
3.36	225.12	28	574	.392	10.976
3.78	253.26	30	615	.412	12.36
4.21	282.07	35	717.5	.393	13.75

B. ANIMAL DATA

Animal Numbers	Time (Min.)	Nominal CXT mg.min	Deaths 48 hours	Initial Animal Weights (g)	Final Animal weights (g)	% Change in Weight 48 hours
1-4	15	4.14	0/4	27.0 ± 1.6	22.0 ± 0.4	- 18.5
20-23	20	9.44	0/4	23.5 ± 0.7	18.7 ± 1.2	- 15.1
24-27	25	10.3	1/4	25.1 ± 0.6	21.3 ± 1.5	- 15.1
28-31	28	10.98	4/4	22.8 ± 0.5	----------	------
16-19	30	12.36	4/4	22.2 ± 1.0	----------	------
5-8	35	13.75	3/4	23.9 ± 0.4	17.1	- 28
9-15	35	-0-	0/6	25.3 ± 2.1	23.7 ± 1.3	- 6%

ANGUIDINE

Nominal Concentration x Time vs. Mortality

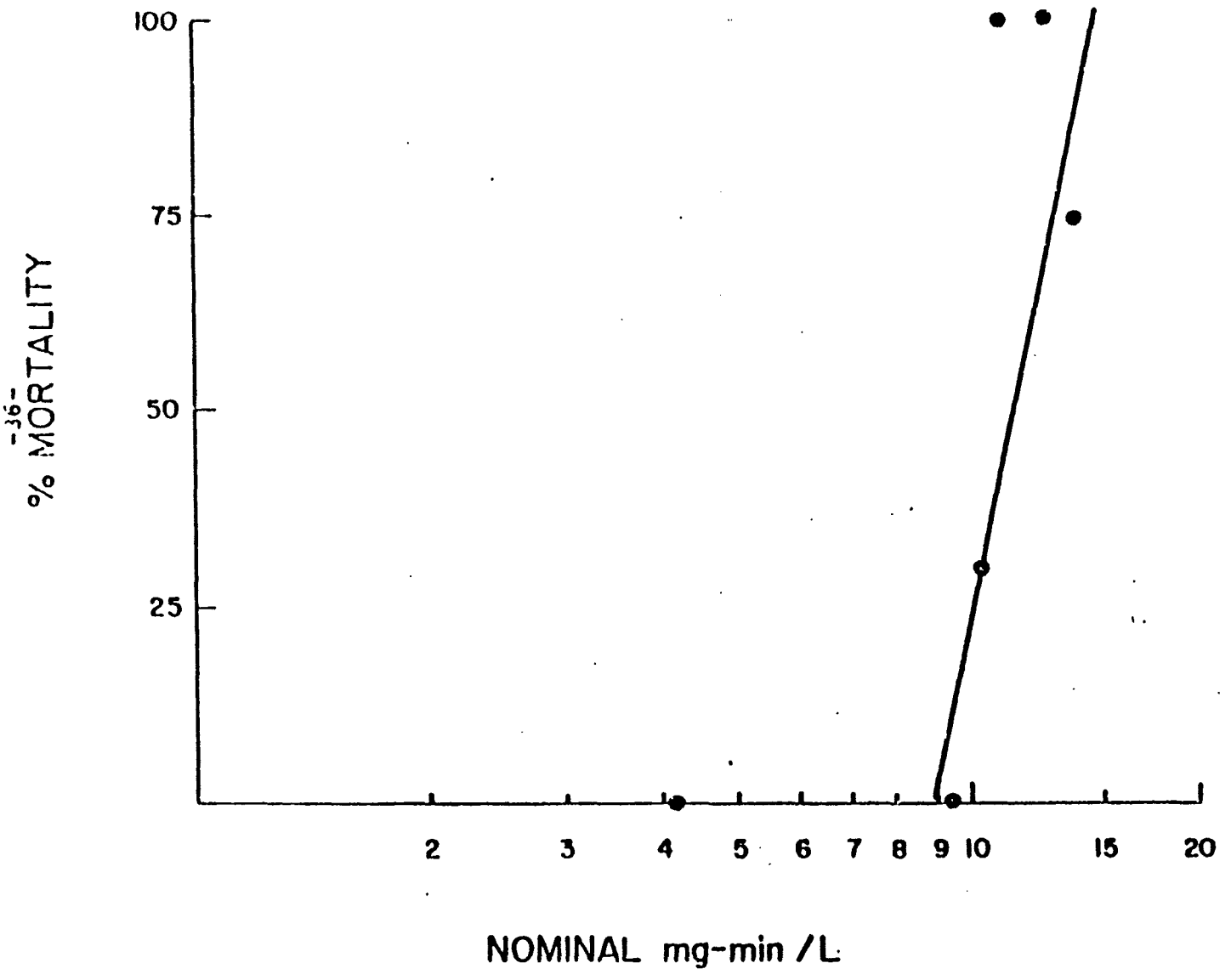


FIGURE 1